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L2: Entry 2 of 2 File: USPT Oct 3, 2000

US-PAT-NO: 6126938
DOCUMENT-IDENTIFIER: US 6126938 A

TITLE: Methods for inducing a mucosal immune response

DATE-ISSUED: October 3, 2000

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
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U.S. PATENT DOCUMENTS

Search Selected | Search ALL

	PAT-NO	ISSUE-DATE	PATENTEE-NAME	US-CL
☐	5529777	June 1996	Andrianov et al.	424/184.1
☐	5538729	July 1996	Czinn et al.	424/234.1
☐	5679564	October 1997	Pace et al.	424/184.1
☐	5833993	November 1998	Wardley et al.	424/199.1
☐	5853763	December 1998	Tice et al.	424/234.1

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FOREIGN PATENT DOCUMENTS

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2220211	January 1990	GB	
9503824	February 1995	GB	
9106282	May 1991	WO	

OTHER PUBLICATIONS

Nedrud et al, Journal of Immunology, 139, 3484-3492, 1987.
McGhee et al, vaccine, 10, 75-88, 1992.
Gallichman et al, Jour. infect. Diseases, 168, 622-629, 1993.

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ABSTRACT:

The invention relates to a pharmaceutical composition intended for inducing in a host mammal a protective immune response against an antigen, at a mucosal effector site, which comprises at least two identical or different products each containing an inducing agent for the immune response, selected from the antigen and, provided the antigen is protein in nature, an expression cassette capable of expressing the antigen, for a concomitant or consecutive administration; one of the products being formulated so as to be administered via the nasobuccal route so that the inducing agent is targeted to the inducer site(s) for an immune response in the naso-oropharynx or the salivary glands, the other product being formulated so as to be administered via a suitable mucosal route other than the nasal route, so that the inducing agent is targeted to the inducer site(s) for an immune response at the effector site at which the immune response is sought. Optionally, such a composition can also comprise a third product, identical to or different from the first two, formulated for systemic administration.

28 Claims, 18 Drawing figures

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Prospects for the Development of a Vaccine Against *Helicobacter pylori*

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Summary

Over 50% of the world population is chronically infected by the gastric pathogen, *Helicobacter pylori*, which is responsible for most peptic ulcer disease and is closely associated with adenocarcinoma of the stomach. Current therapies for peptic ulcer disease include antibiotic eradication of *H. pylori* infection. While effective, the high cost, difficulty of patient compliance with the treatment regimens, and risks of selection for resistant strains make these therapies impractical on a large scale. Studies of the pathogenesis of *H. pylori* have led to the identification of bacterial antigens as candidates for inclusion in novel vaccines against this disease. Both prophylactic and therapeutic vaccination have been demonstrated in animal models of *Helicobacter* infection. Preclinical evaluations of several antigens are at present under way and trials of vaccination in humans are planned.

By now, very few people doubt that *Helicobacter pylori* infection is the major cause of most type B gastritis and peptic ulcer disease.[1] The bulk of the evidence for this comes from epidemiological data which effectively demonstrate that if you do not have the infection, you do not get the disease. In addition, 3 cases of voluntary or accidental ingestion of live *H. pylori* cultures have been reported.[2-4] In these cases, infection was followed by severe gastritis that became chronic and was resolved only on antibiotic eradication of the bacteria. Finally, several trials have demonstrated that ulcer relapse after healing with antacid drugs can be reduced from 80% in 2 years to a few percent when treatment includes eradication of infection.[5]

These data have led the US Food and Drug Administration to consider eradication of infection as a surrogate for reduction of ulcer relapse in clinical trials of anti-*H. pylori* therapies. Not to be discounted also is the accumulating body of evidence that *H. pylori* infection is closely associated with gastric adenocarcinoma and B cell MALT lymphoma.[6,7]

Although the gastric niche occupied by *H. pylori* presents problems of drug delivery and stability, several regimens of combined antibiotics have proven effective in eradication of infection, with success rates of more than 90% reported.[5] Patient compliance with the complex and often unpleasant treatment is, however, a major problem. Nevertheless, in the long term, given the persistence of disease in the population, widespread use of this approach will inevitably lead to the selection of antibiotic-resistant strains of *H. pylori* and other important human pathogens. Hence, there is an urgent need for an effective vaccine against *H. pylori* infection.

1. Identification of Candidate Antigens

Modern approaches to vaccine design involve the identification of protective antigens which can be obtained in highly purified, nontoxic forms, hence eliminating the risk of reversion to virulence

of the organism and/or more or less severe adverse effects. In the design of vaccines against bacterial pathogens, protective antigens are generally sought among the virulence factors involved in bacterial colonisation and persistence or those involved in induction of disease. Colonisation factors identified in *H. pylori* to date include the bacterial urease[8,9] which, in hydrolysing urea, produces ammonia and carbon dioxide which help buffer the stomach acid; the flagella,[10] which are required for mobility in the gastric mucus; and adhesion molecules required for attachment of the bacteria to the gastric epithelium.[11,12]

In addition to these factors which are expressed by all strains of *H. pylori* so far isolated, some strains produce a potent cytotoxin (VacA)[13] which is believed to play a major role in pathogenesis.[14] Expression of the most toxic form of this protein appears to be restricted to a subset of *H. pylori* strains which differ genetically from the nontoxic isolates.[15] These strains are characterised by the expression of an immunodominant surface antigen coded for by the cytotoxin-associated gene A (*cagA*).[16] This gene forms part of a large chromosome fragment which is not found in the genome of the nontoxic strains.[16]

Overwhelming epidemiological evidence has associated infection with CagA-expressing strains with the more severe forms of disease including peptic ulcer and gastric cancer.[17,18] We have suggested that CagA-expressing, potently toxic isolates of *H. pylori* be referred to as 'type I' strains to distinguish them from strains lacking these properties, which could be referred to as 'type II'.[15,19] Although the recently emerging complexity in *H. pylori* genetics[20] has raised questions as to the validity of this classification, particularly the discovery of strains of intermediate phenotype,[15] we feel that the distinction continues to be useful and satisfactory, and that further knowledge of *H. pylori* pathogenesis will support classification of strains along these lines. Hence, for the purposes of this article, we refer to CagA-expressing, potently toxic strains as type I, and those nontoxic, CagA-negative strains as type II.

Evidence for a direct role for the cytotoxin in pathogenesis comes from studies in mice. Intragastric administration of extracts of type I, CagA-positive, cytotoxic strains of *H. pylori* caused severe epithelial erosion, ulceration and inflammatory cell infiltration into the lamina propria. Administration of extracts of type II, nontoxic strains caused only mild gastritis.[21] Administration of highly purified, active VacA caused epithelial erosion similar to that caused by the extracts but did not result in inflammatory cell infiltration.

These results have been confirmed in a mouse model of *H. pylori* infection which produces pathology similar to that seen in human disease.[22] In particular, infection with a genetically altered type I strain in which the cytotoxin gene had been ablated caused considerably milder epithelial damage than the parental toxic strain, but still caused significant inflammation (Ghiara & Telford, unpublished observations).

Hence, VacA is likely to play a major role in the epithelial damage caused by *H. pylori* infection of humans and as such, after suitable detoxification, is a major candidate for inclusion in a vaccine against *H. pylori*–associated disease. On the other hand, other factors appear to be involved in the massive inflammation associated with infection with type I strains. Recent evidence indicates that the products of genes expressed only by these strains contribute to the production of factors which induce interleukin-8 (IL-8) expression in gastric epithelial cells.[23] This cytokine is a potent neutrophil chemoattractant and may be involved in inflammatory cell infiltration. Furthermore, the CagA protein itself appears to be a dominant antigen for CD4+ T cells isolated from gastric biopsies of peptic ulcer patients (D'Elios, Del Prete & Telford, unpublished observations). The majority of the CagA-specific clones isolated were of the T helper cell-1 (T_H1) type which produce tumour necrosis factor-α (TNFα) and interferon-γ (IFNγ), both potent inflammatory cytokines.

2. Assessing Vaccine Candidates

Chen et al.[24] first showed the potential for vaccination against *Helicobacter* infection using a mouse model of infection with the related species *H. felis*. These authors demonstrated that oral immunisation with lysates in combination with cholera toxin induced protection against infection with this species of *Helicobacter*. This was an important result in that it demonstrated proof of concept for vaccination against an organism which is capable of colonising the host without inducing a protective immune response. Hence, doubts that protective immunity could be induced by vaccination where live infection failed were laid aside.

The use of cholera toxin as a mucosal adjuvant probably plays an important role in the induction of protective immunity, since administration of lysate alone failed to induce protection. Protein antigens are generally poorly immunogenic when administered orally due to the intrinsic tolerance of the gastrointestinal system to ingested substances. Cholera toxin, and the very closely related heat-labile toxin from enteropathogenic *Escherichia coli*, however, not only are highly immunogenic when administered orally but also confer immunogenicity to coadministered antigens.[25] These toxins thus appear to be able to break oral tolerance.

Subsequently,[26-28] it has been demonstrated that purified *H. pylori* urease could induce protection against *H. felis* infection when administered with cholera toxin. This was the first demonstration that a purified antigen was potentially useful as an anti-*Helicobacter* vaccine.

While the *H. felis* model was important for these initial demonstrations, the fact that this species does not adhere to the gastric epithelium, lacks important virulence factors such as CagA and VacA, and does not cause ulceration, limited its usefulness in studying *H. pylori* pathogenesis and the potential for vaccination.

More recently, Marchetti et al.[22] have adapted fresh isolates for *H. pylori* to chronic infection of mice by serial passaging. These adapted strains colonise mice efficiently and adhere to the gastric epithelium. As mentioned in section 1, colonisation with type I CagA, VacA-positive strains causes epithelial erosion and inflammation similar to that seen in *H. pylori*–associated disease in humans. This model is rapidly becoming a new standard for assessment of vaccine candidates.

To date, several purified antigens have been shown to induce protective immunity in this model. As in the *H. felis* model, immunisation with urease induces efficient protection. In addition, purified VacA,[22] recombinant VacA, CagA and 60 kDa heat shock protein (Hsp60)[29] also induce protection in this model when administered with either cholera toxin or heat-labile toxin (table I).

Combinations of purified antigens have also been tested. With a combination of recombinant urease (B subunit) and heat shock protein A (HspA), complete protection against *H. felis* infection has been achieved in the mouse.[30] Moreover, an association of VacA and native purified urease was able to confer full protection against infection of mice by *H. pylori* (table I).

3. Therapeutic Immunisation

The encouraging results from the prophylactic vaccination experiments clearly demonstrated that, using appropriate immunisation techniques, an immune response capable of preventing colonisation was possible, whereas natural infection failed to elicit the appropriate response.

This raised the question of whether a protective immune response could be induced in an animal already chronically infected by the bacteria. If so, the induced immunity could potentially eliminate the bacteria. Several series of experiments (table II) have now in the main validated this hypothesis.[31,32] Bacterial lysate[29] and purified antigens such as recombinant VacA have also been shown to eradicate *H. pylori* from chronically infected mice and to induce protective memory against a subsequent reinfection (Ghiara & Marchetti, unpublished observations; Corthesy-Theulaz et al.[31]) [table II].

The concept of therapeutic vaccination is entirely new and was not predictable. Vaccination has traditionally been considered as a prophylactic

Table I. Prophylactic vaccination against *Helicobacter pylori*. Current status of mouse studies

H. pylori antigen	Adjuvant	Infection	Protection (%)	Reference
Sonicate	CT, LT, LTK63	*H. pylori*	80-100	22,29
	CT, LT	*H. felis*	≈70	28
Urease (Ure)[a]	LT, CT, LTK63	*H. pylori*	≈80	22,29
	LT, CT	*H. felis*	70	28,33
UreB subunit	LT, CT	*H. felis*	25-70	27,28
HspA	LT, CT	*H. felis*	50	30
HspB	LT, LTK63	*H. pylori*	≈50	Marchetti et al., unpublished data
	LT, CT	*H. felis*	≈50	30
UreB + HspA	LT	*H. felis*	100	30
VacA	LT, CT, LTK63	*H. pylori*	≈80	22,29
CagA	LTK63	*H. pylori*	≈70	Marchetti et al., unpublished data
VacA + Urease[a]	LT, LTK63	*H. pylori*	100	29

a Holoenzyme.

Abbreviations: CagA = cytotoxin-associated gene A; CT = cholera toxin; Hsp = heat shock protein; LT = heat-labile enterotoxin of *Escherichia coli*; LTK63 = genetically detoxified mutant of LT.

measure. In fact, for some organisms, for example *Mycobacterium*, vaccination of already infected individuals has been considered potentially dangerous. The success obtained in the animal model of *H. pylori* infection indicates that if the immune system can be stimulated in the correct way against important antigens, a therapeutic response can be induced.

4. What Is a Protective or Therapeutic Immune Response Against *H. pylori*?

Little is understood about the induction and the nature of mucosal immune responses. Whereas the efficacy of most commercially available vaccines can be predicted by their capacity to induce a specific serum immunoglobulin response, this does not seem to be the case for *H. pylori* since natural infection induces strong serum responses in the absence of protection. On the other hand, secretory immunoglobulin (Ig)A may play an important role in protection from infection of mucosal surfaces. In fact, a correlation has been established between specific secretory IgA levels against urease antigen and protection.[133]

Whether this is the major mechanism of protection or whether other aspects of the immune system, such as IgG from mucosa-infiltrated B cells or cell-mediated responses, play a role remains to be determined. Further comparisons of the details of the immune responses induced by infection or vaccination are required.

5. Safe Mucosal Adjuvants

To date, cholera toxin and *E. coli* heat-labile toxin are the only known mucosal adjuvants capable of conferring antigenicity on orally adminis-

Table II. Therapeutic vaccination against *Helicobacter pylori*: current status of mouse studies

Infection	*H. pylori* antigen	Adjuvant	Eradication (%)	Reference
H. felis	Sonicate	CT	70-90	32
	Urease B sububit	CT	≈50	31
H. pylori	Sonicate	LTK63	70	29, Ghiara & Marchetti, unpublished data
	Recombinant VacA	LTK63	90	29, Ghiara & Marchetti, unpublished data

Abbreviations: CT = cholera toxin; LTK63 = genetically detoxified mutant of heat-labile enterotoxin of *Escherichia coli*.

tered antigens. The toxicity of these molecules, however, effectively prevents their use in humans. Early data indicated that the B subunit of cholera toxin lacking the enzymatically active subunit might function as an adjuvant, but subsequent experiments showed that the adjuvancy of commercially available preparations of B subunit was due to contamination by small quantities of active toxin.[34,35]

More recently, genetically detoxified mutants of *E. coli* toxin have been made which completely lack toxic activity but retain the capacity to confer antigenicity to model antigens.[36] One of these mutants (LTK63), which has a single amino acid substitution in the active site of the enzyme, has been tested in both prophylactic and therapeutic vaccination experiments against *H. pylori* in the mouse model with notable success (tables I and II). The nontoxic mutant functioned equally as well as the wild type toxin as an adjuvant for the urease, CagA and VacA antigens, opening the way for the testing of these molecules in humans.

6. Future Perspectives

The major question still unanswered is: will oral vaccination against *H. pylori* also work in humans? The vaccination of mice against both *H. felis* and the human pathogen *H. pylori* has been successful, and the results are encouraging. Nevertheless, there are significant differences between the mucosal immune systems of mice and humans. Will the detoxified adjuvants work in the human immune system? These questions and others relating to the final formulation of an effective *H. pylori* vaccine will only be answered by clinical trials with human participants.

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Activation of Human THP-1 Cells and Rat Bone Marrow-Derived Macrophages by *Helicobacter pylori* Lipopolysaccharide

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The mechanism by which *Helicobacter pylori*, which has little or no invasive activity, induces gastric-tissue inflammation and injury has not been well characterized. We have previously demonstrated that water-extracted proteins of *H. pylori* are capable of activating human monocytes by a lipopolysaccharide (LPS)-independent mechanism. We have now compared activation of macrophages by purified LPS from *H. pylori* and from *Escherichia coli*. LPS was prepared by phenol-water extraction from *H. pylori* 88-23 and from *E. coli* O55. THP-1, a human promyelomonocytic cell line, and macrophages derived from rat bone marrow each were incubated with the LPS preparations, and cell culture supernatants were assayed for production of tumor necrosis factor alpha (TNF-α), prostaglandin E_2 (PGE_2), and nitric oxide. THP-1 cells showed maximal activation by the LPS molecules after cell differentiation was induced by phorbol 12-myristate 13-acetate. Maximal TNF-α and PGE_2 production occurred by 6 and 18 h, respectively, in both types of cells. In contrast, NO was produced by rat bone marrow-derived macrophages only and was maximal at 18 h. The minimum concentration of purified LPS required to induce TNF-α, PGE_2, and NO responses in both types of cells was 2,000- to 30,000-fold higher for *H. pylori* than for *E. coli*. Purified LPS from three other *H. pylori* strains with different polysaccharide side chain lengths showed a similarly low level of activity, and polymyxin B treatment markedly reduced activity as well, suggesting that activation was a lipid A phenomenon. These results indicate the low biological activity of *H. pylori* LPS in mediating macrophage activation.

Helicobacter pylori causes persistent infection of the human stomach and is now recognized as the most common cause of chronic superficial gastritis (1). Although usually asymptomatic, *H. pylori*-induced chronic gastritis is an important risk factor for the development of peptic ulcer disease and adenocarcinoma of the stomach (12, 24), and consequently this lesion is clinically important. However, intense inflammation may lead to loss of gastric glandular structure and function, and with the development of atrophic gastritis, the ecological niche for *H. pylori* is progressively lost (13). Thus, there exists selective pressure for *H. pylori* to modulate induction of tissue injury (3).

At present, much about the pathogenesis of *H. pylori*-induced gastric inflammation and injury is not well understood. Although the organism does not invade the lamina propria, it induces an infiltrate with T-lymphocytes, plasma cells, mononuclear phagocytes, and neutrophils (26, 34), and expression of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukins-1, -6, and -8, also is enhanced (6, 27). We have previously demonstrated that water-extracted proteins of *H. pylori* are chemotactic for human polymorphonuclear leukocytes and monocytes (17) and also activate these cells by a lipopolysaccharide (LPS)-independent mechanism (18). For many other bacterial species, LPS is a powerful activator of mononuclear phagocytes (31, 42).

LPS is a major component of the outer membranes of gram-negative bacteria (15), with a lipid core and polysaccharide side chains of variable length. The carbohydrate portion contains a core region which usually is genus or species specific and an outermost chain that is strain specific and forms the basis of the O-antigenic classification (16). The lipid portion (lipid A) represents the endotoxic principle of biologically active LPS (9). LPS from members of the family *Enterobacteriaceae* has been demonstrated to be highly proinflammatory (31), and LPS from *Escherichia coli* and *Salmonella* spp. is representative of the group. Compared with the lipid A of members of the *Enterobacteriaceae*, the lipid A of *H. pylori* has an unusual composition of fatty acids (10) and also a different phosphorylation pattern, with 1′- but not 4′-phosphate present in the backbone of lipid A D-glucosamine disaccharide (21). *H. pylori* LPS has been reported to be biologically less active than LPS from members of the *Enterobacteriaceae* when mitogenicity, pyrogenicity, and lethal toxicity were compared in in vivo assays (23).

The purpose of the present study was to compare the ability of purified LPS from *H. pylori* and *E. coli* to activate monocytic cells in vitro. We hypothesized that evolutionary pressure for *H. pylori* persistence in the stomach (3) would select for LPS molecules with relatively low activity.

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MATERIALS AND METHODS

Cell line. THP-1 (ATCC TIB202), originally isolated from a child with acute leukemia, are mature cells in the monocyte/macrophage lineage with a normal

TABLE 1. Chemical composition of whole-cell and LPS preparations from *H. pylori* and *E. coli* strains

Strain	Whole cell[a]			LPS preparation[a]			Ratio B/A
	Protein concn (mg/ml)	KDO concn (mg/ml)[b]	Ratio A[c]	Protein concn (mg/ml)	KDO concn (mg/ml)	Ratio B[c]	
H. pylori 88-22	7.4	0.013	0.002	0.23	0.009	0.04	20
H. pylori 88-23	4.8	0.013	0.003	0.33	0.011	0.03	10
H. pylori 84-182	2.4	0.007	0.003	0.17	0.005	0.03	10
H. pylori 84-183	5.5	0.009	0.002	0.18	0.011	0.06	30
E. coli O55	ND[d]	ND	ND	0.05	0.027	0.54	ND

[a] All values shown are the means of two separate determinations.
[b] KDO, 2-keto-3-deoxyoctonate.
[c] Ratios A and B were calculated as the proportions of KDO to protein in whole cells (ratio A) and in purified LPS (ratio B).
[d] ND, not determined.

diploid karyotype (38), and they produce TNF-α and other cytokines in response to purified endotoxin (20). These nonadherent cells were maintained in continuous culture with RPMI 1640 (GIBCO/BRL, Grand Island, N.Y.), 10% fetal bovine serum (GIBCO/BRL), and 0.05 mM 2-mercaptoethanol (GIBCO/BRL) in an atmosphere of 5% CO_2 at 37°C. The doubling time for these cells under these conditions is approximately 48 h. THP-1 cells were treated with phorbol 12-myristate 13-acetate (Calbiochem Co., La Jolla, Calif.) to induce maturation of the monocytes and became macrophage-like; differentiated macrophages were identified by morphological features and their ability to adhere to plastic, as described elsewhere (37). Before experimentation or treatment with phorbol 12-myristate 13-acetate, THP-1 cells were washed three times with culture medium without fetal bovine serum and resuspended to a concentration of 10^6 cells per ml. Cell viability was determined to be >95% by the trypan blue dye exclusion method (35).

Rat bone marrow-derived macrophage culture. Rat bone marrow macrophages were obtained from precursor bone marrow cells as described previously (30). Femoral bone marrow cells were grown in a 150-mm tissue culture dish at 5×10^7 cells per ml for 6 days in 50 ml of culture medium (Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and penicillin-streptomycin) plus 10% L-cell-conditioned medium as previously described (38). The mature rat bone marrow macrophages were removed from the dishes with cold 5 mM EDTA in phosphate-buffered saline (PBS) and replated into 24-well culture plates (Sarstedt, Inc. Newton, N.C.) at 5×10^5 cells per ml. They were incubated at 37°C in 5% CO_2 in the presence of different concentrations of the bacterial products and controls. Aliquots were obtained at 0, 6, and 18 h of incubation, and the supernatants were collected and either tested immediately or stored at −70°C.

Bacterial strains and culture conditions. *H. pylori* 84-183, 84-182, 88-22, and 88-23, clinical isolates in the Vanderbilt *Campylobacter/Helicobacter* culture collection (5, 28), were stored at −70°C until use. Bacteria were inoculated onto Trypticase soy agar containing 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.) and incubated for 48 to 72 h in ambient atmosphere plus 5% CO_2. Bacteria were suspended in sterile pyrogen-free saline and centrifuged at 3,000 × *g* for 20 min, and the pellet was resuspended in distilled water.

LPS preparations. The LPS from the four *H. pylori* strains was prepared by the hot-phenol–water method of Westphal and Jann (41), and subsequent purification steps were performed essentially as described by Daniels et al. (7). In brief, bacterial cells from blood agar plates were scraped into saline, centrifuged (5,000 × *g* for 15 min), and resuspended in water with an equal volume of 90% phenol at 60°C for 15 min. After the mixture was cooled to 10°C and centrifuged (10,000 × *g* for 20 min), the aqueous layer was removed. This extraction procedure was repeated twice, and the pooled water-extracted layers were dialyzed for 48 h against several changes of water and lyophilized. As a control, LPS purified by the hot-phenol–water method from *E. coli* O55:B5 (List Biological Laboratories, Inc., Campbell, Calif.) was used in each experiment. In several experiments, the LPS from *E. coli* and *H. pylori* strains were treated with polymyxin B (Sigma Chemical Co., Saint Louis, Mo.) to determine the effect of binding to lipid A on the biological activities of these molecules (22). Preparations of LPS were preincubated for 1 h at 37°C with twofold concentrations of polymyxin B, and the activity was assayed in the systems used for the untreated LPS.

Analytical methods. Protein concentrations were measured by the bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.). For determination of 2-keto-3-deoxyoctonate concentrations, the thiobarbituric acid method (39) with the modifications described by Keleti and Lederer (14) was used with 3-deoxyoctulosonic acid ammonium salt (Sigma) as the standard.

Monokine release. Aliquots of purified *H. pylori* or *E. coli* LPS were added in duplicate to THP-1 cells (10^6 cells per ml) in 24-well tissue culture plates or to rat bone marrow macrophages (5×10^5 cells per ml) and then incubated at 37°C in a 5% CO_2 atmosphere. Cell suspensions obtained at 0, 6, and 18 h after incubation were transferred to 1.5-ml polypropylene tubes and centrifuged at 12,000 × *g* for 3 min at room temperature, and the supernatants were stored at −70°C until the monocyte-derived factors could be assayed.

Measurement of TNF-α. Biological activity of TNF-α was measured by an assay of L929 fibroblast lysis, as previously described (4). Briefly, L929 cells (5×10^5 cells per ml) in alpha minimal essential medium (GIBCO BRL) with 10% fetal bovine serum were incubated overnight at 37°C with 5% CO_2 in 96-well flat-bottom plates (Sarstedt). An aliquot of 100 μl of fresh alpha minimal essential medium plus 15 μg of actinomycin D (Sigma) per ml and 100 μl of the cell culture supernatant were added, and the plates were again incubated overnight at 37°C with 5% CO_2. Subsequently, plates were washed with Dulbecco's PBS and cells were stained with 0.1% crystal violet in 100% methanol. The number of remaining cells as assessed by A_{540} was used to determine cell lysis; purified TNF-α was used as a positive control and for development of a standard curve.

Nitric oxide synthase assay. Nitric oxide activity was measured as nitrite production (19) in stimulated rat bone marrow-derived macrophages and THP-1 monocytes; each assay was performed in triplicate. Nitrite was measured by mixing 100 μl of the cell-free culture supernatant with an equal volume of Griess reagent (1 part of 0.1% naphthylethylenediamine dihydrochloride to 1 part of 1% sulfanilamide in 5% phosphoric acid) (11). The A_{550} was determined, and the nitrite concentration was calculated from a standard curve with sodium nitrite as the reference.

PGE_2 assay. Prostaglandin E_2 (PGE_2) production in cell supernatants was determined by a gas chromatography-mass spectrometry method involving stable isotope dilution techniques with [$^2H_4$]PGE_2 as an internal standard, as previously described (25).

RESULTS

Chemical analysis of purified LPS. The four strains were selected because the LPS profile for each was different as observed by polyacrylamide gel electrophoresis (PAGE) analysis (28). However, the chemical analysis of the four purified LPS preparations from these *H. pylori* strains showed similar protein and 2-keto-3-deoxyoctonate contents (Table 1). In total, protein contamination was less than 5%. In most of the subsequent studies, the preparation from strain 88-23 was used.

Activation response to LPS in undifferentiated and differentiated THP-1 cells. We first evaluated the ability of LPS to activate either differentiated or undifferentiated THP-1 cells. LPS preparations from both *H. pylori* and *E. coli* were able to activate both types of cells to induce the production of TNF-α and PGE_2 (Table 2). However, undifferentiated THP-1 cells were 1.5×10^5-fold less responsive to *E. coli* LPS than were differentiated THP-1 cells, as assessed by production of TNF-α. The effects of *E. coli* LPS on macrophages are markedly enhanced by an LPS-binding protein present in serum (43). The poor response obtained with *E. coli* LPS in undifferentiated THP-1 cells is at least in part because no serum source (and thus no LPS-binding protein) was added to the medium. When PGE_2 was used as the indicator of activation, undifferentiated cells were $>8 \times 10^3$-fold less sensitive than differentiated cells to activation by *E. coli* LPS (Table 2). The undifferentiated cells also were poorly activated by *H. pylori* LPS. In contrast, the differentiated THP-1 cells were markedly responsive to LPS from *E. coli* but poorly activated by *H. pylori* LPS.

TABLE 2. Minimal concentrations of LPS from *H. pylori* or *E. coli* inducing responses in undifferentiated and differentiated THP-1 cells

LPS source	Concn of LPS (μg/ml)			
	Undifferentiated		Differentiated	
	TNF-α[a]	PGE_2[b]	TNF-α	PGE_2
H. pylori	10	2.5	0.15	2.5
E. coli	>50	2.5	0.0003	0.0003

[a] Culture supernatants were obtained after a 6-h incubation of the THP-1 cells with differing concentrations of LPS. A positive response was defined as TNF-α induction of ≥0.07 μg/ml. This value represents twice the concentration of TNF-α obtained at baseline. Results shown are means of two replicate determinations.

[b] Culture supernatants were obtained after 24 h of incubation of the THP-1 cells with different concentrations of LPS. Response was defined as PGE_2 induction of ≥100 pg/ml; this value represents the concentration of PGE_2 obtained as baseline. Results shown are means of two replicate determinations.

On the basis of these preliminary results, the remainder of the experiment focused only on differentiated THP-1 cells.

Minimal LPS concentration to induce activation in differentiated THP-1 cells. In a second series of experiments, the differentiated THP-1 cells again were much more responsive to LPS from *E. coli* than from *H. pylori* (Table 3). Results for the commercial preparation from an *E. coli* O55 strain and a preparation from an O157 strain made in this laboratory showed nearly identical results in the range from 1 ng to 1 μg (data not shown). The minimal concentration required to activate the THP-1 cells was between 2×10^3- and 30×10^3-fold lower for *E. coli* than *H. pylori* LPS. However, as expected (36), both LPS preparations failed to induce nitric oxide production in this system.

Minimal LPS concentrations to induce activation in rat bone marrow macrophages. We then compared the ability of the *E. coli* and *H. pylori* LPS preparations to induce responses in rat bone marrow macrophages. Induction of nitric oxide, TNF-α, and PGE_2 responses were used as markers of activation. In all instances, *E. coli* LPS was a substantially better activator than was *H. pylori* LPS. Depending on the assay, 2×10^3- to 20×10^3-fold less *E. coli* LPS was required to induce the same level of responses than was *H. pylori* LPS (Table 4).

Comparison of *H. pylori* LPS preparations inducing activation. We then compared the relative ability of purified LPS preparations from four *H. pylori* strains to induce activation in rat bone marrow macrophages and differentiated THP-1 cells (Table 5). The results indicated that the minimal concentrations required to induce responses in macrophages were high

TABLE 3. Minimum concentrations of LPS from *H. pylori* or *E. coli* inducing responses in differentiated human THP-1 cells

Indicator of activation	Concn of LPS (μg/ml)		Fold difference
	E. coli	*H. pylori*	
TNF-α[a]	0.00007	2.5	3.6×10^4
NO[b]	>10	>10	NA[c]
PGE_2[d]	0.0003	0.6	2×10^3

[a] Response is defined as TNF-α induction of ≥0.07 ng/ml, as indicated in Table 2. Results shown are means of two replicate experiments.

[b] After 24 h of incubation of the THP-1 cells with differing concentrations of LPS, culture supernatant was used for determinations. Response was defined as NO induction of ≥ 0.001 nM NO_2 per ml. Results shown are means of two replicate experiments.

[c] NA, not applicable.

[d] Response is defined as PGE_2 induction of ≥100 pg/ml, as indicated in Table 2. Results shown are means of two replicate experiments.

TABLE 4. Minimum concentrations of LPS from *H. pylori* or *E. coli* that induce responses in rat bone marrow macrophages

Indicator of activation	Concn of LPS (μg/ml)		Fold difference
	E. coli	*H. pylori*	
TNF-α[a]	0.3	600	2×10^3
NO[b]	0.015	300	2×10^4
PGE_2[c]	0.3	2,500	8×10^3

[a] Response is defined as TNF-α induction of ≥0.07 ng/ml, as indicated in Table 2. Results are means of two replicate experiments.

[b] Response is defined as NO induction of ≥0.001 nM NO_2 per ml, as indicated in Table 3. Results are means of two replicate experiments.

[c] Response is defined as PGE_2 induction of ≥100 pg/ml, as indicated in Table 2. Results are means of two replicate experiments.

for all four strains, with only small (≤fourfold) differences. These data indicate that the macrophage-activating constituents of *H. pylori* LPS are conserved and thus suggest that these structures may be present in the lipid A moiety.

Polymyxin B blocks LPS-induced TNF-α production. By binding to lipid A, polymyxin B is a well-known inhibitor of activation properties of LPS from the members of the *Enterobacteriaceae* (22). To determine whether the effect of polymyxin B on *H. pylori* LPS is similar, we preincubated different concentrations of *H. pylori* LPS, with or without polymyxin B, before adding these preparations to THP-1 cells. Preincubation with polymyxin B markedly inhibited the ability of *H. pylori* LPS to induce TNF-α release (Fig. 1). These results indicate that the basis for activation of macrophages by *H. pylori* LPS is lipid A mediated and that polymyxin neutralizes this activity, despite the differences in lipid A structure in comparison with members of the *Enterobacteriaceae* (10, 21).

DISCUSSION

The cardinal lesion of *H. pylori* colonization of the stomach is gastric inflammation (1), but since these organisms do not invade tissue, the proinflammatory effects of superficial or released bacterial products are of interest. Bacterial LPSs are classic mediators of inflammation because of their activation of phagocytic cells, endothelial and epithelial cells, and lymphocytes (32). However, despite a general conservation of LPS structure, large differences in their proinflammatory activity have been noted (33).

Thus, it is reasonable to explore whether the LPS of *H. pylori* is involved in induction of the characteristic inflammatory in-

TABLE 5. Minimum concentrations of LPS from four *H. pylori* strains that induce responses in macrophages

H. pylori strain	Concn of LPS (μg/ml) in:					
	Rat bone marrow macrophages			Differentiated THP-1 cells		
	NO[a]	TNF-α[b]	PGE_2[c]	NO	TNF-α	PGE_2
88-23	2.5	0.6	2.5	>10	2.5	2.5
88-22	2.5	2.5	2.5	>10	0.6	0.6
84-182	0.6	2.5	2.5	>10	2.5	0.6
84-183	2.5	2.5	2.5	>10	0.6	2.5

[a] Response is defined as NO induction of ≤0.001 nM NO_2 per ml, as indicated in Table 3. Results are means of two replicate experiments.

[b] Response is defined as TNF-α induction of ≥0.07 ng/ml, as indicated in Table 2. Results are means of two replicate experiments.

[c] Response is defined as PGE_2 induction of ≥ 100 pg/ml, as indicated in Table 2. Results are means of two replicate experiments.

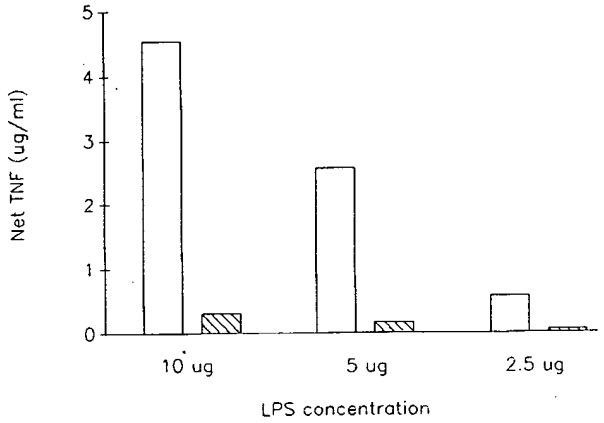

FIG. 1. Effect of polymyxin B on *H. pylori* LPS-induced TNF-α production by THP-1 cells. Empty bars, LPS without polymyxin B treatment; hatched bars, LPS treated with a twofold concentration of polymyxin B. The different LPS concentrations were incubated with or without polymyxin B at 37°C for 1 h, and then the preparations were incubated with THP-1 cells for 6 h. Culture supernatants were harvested and TNF-α concentrations were determined as described in Materials and Methods. Results shown are the means of two replicate determinations.

filtrate. Previous studies have shown that *H. pylori* LPS is substantially less active than LPS from members of the *Enterobacteriaceae* in a variety of biologic activities including rabbit pyrogenicity, B-cell mitogenicity, and ability to gel *Limulus* lysates (10, 21, 23). However, interactions with phagocytic cells were not addressed.

In our studies, *H. pylori* LPS was substantially less stimulatory to macrophages than was *E. coli* LPS. In studies of two different types of cells and assays of three different products of activation, the results are highly consistent. This multiplicity of approaches increases our confidence that the observation is correct. Furthermore, we have also performed experiments with *E. coli* O157 LPS that was prepared in our laboratory by the Westphal technique, and the results were similar to those observed for the commercially obtained *E. coli* O55 LPS. Although *H. pylori* strains may show marked differences in LPS profiles by PAGE (28), their abilities to activate macrophages were highly similar. This observation suggests that as with other gram-negative organisms, the ability to activate macrophages is a function of conserved core structures. The studies with polymyxin B confirm the important role of lipid A in this phenomenon. Our observations are consistent with results of previous studies indicating its low level of biological activity (9, 15, 16) and suggest that *H. pylori* lipid A features, including long-chain fatty acids and the lack of a phosphate group (21), may be responsible. *Bacteroides fragilis* is another gram-negative organism that is a persistant colonizer of the human gastrointestinal tract (8), and similar to *H. pylori*, its LPS is a poor activator of macrophages (40).

Among individual persons infected with *H. pylori*, there are differences in both degree of inflammation and clinical outcome of infection (29). The LPS structure is one of the few phenotypes of *H. pylori* that shows diversity. However, we found no substantial differences in proinflammatory activity despite this diversity, suggesting that LPS differences do not explain divergent outcomes of infection.

One question raised by our findings is why the LPS of *H. pylori* shows such low proinflammatory activity. Our experimental data are consistent with the general observation that toxicity is a function of the core lipid A moiety, which is likely to be highly conserved. One explanation is that there is selective pressure on *H. pylori* cells to minimize proinflammatory activities to permit long-term colonization (3), since enhanced inflammation, leading to atrophic gastritis, would lead to loss of niche (13). *H. pylori* and *B. fragilis* may be analogous in their requirement for maintaining a low profile at baseline to ensure persistence. Despite the low-level LPS activity, *H. pylori* possesses proteins that are highly efficient in recruiting and activating inflammatory cells (17, 18). Since *H. pylori* may require inflammation to provide a source of nutrients (2), the combination of a constitutively expressed LPS with low-level activity and inducible proteins with high-level activity may be beneficial. Tight regulation of proinflammatory activities could be a mechanism selected by *H. pylori* to maximize the duration of colonization.

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DIGESTIVE BACTERIAL OVERGROWTH (BO) AND HIV INFECTION: A PILOT STUDY. J.Ph. Chave, J. Thorens, F. Frochlich, K. Gyr*, J.J. Gonvers, M.P. Glauser, J. Bille, M. Fried. University Hospitals, Lausanne and *Basle, Switzerland.

Impaired gastric acid secretion and alterations in mucosal immunity have been reported in HIV infection, both theoretically predisposing to BO. We assessed the prevalence of BO in HIV outpatients (pts), applying a newly developed method which permitted sterile sampling. Methods: 24 consecutive HIV+ pts were examined. Pts receiving antibiotic therapy (except aerosolized pentamidine) or antacids/anti-H2/omeprazole were excluded. There were 10 homosexual men, 10 IV drug abusers and 4 heterosexuals. Median CD4+ cells count was 206/mm³ (range: 18-1'743). No pt presented with digestive symptoms. The control group consisted of 34 HIV negative pts referred for suspected peptic ulcer disease. A sterilized double-sheathed tube was passed through the endoscope suction channel and gastric and duodenal juices were aspirated separately under direct visual control. Specimens were plated quantitatively for both aerobic and anaerobic organisms. Parasitological evaluation was done on duodenal samples. BO was defined by total bacterial counts of $>10^5$ CFU/ml. Results: BO was documented in 7/24 HIV+ pts: 4/11 with CD4 <200, 1/7 with CD4 >200<500 and 2/6 with CD4 >500 (3 pts had both gastric and duodenal BO). BO was found in 3/34 control pts ($p<0.05$).

Bact.Counts	$<10^4$		10^4-10^5		BO ($>10^5$)	
(CFU/ml)	HIV+	Controls	HIV+	Controls	HIV+	Controls
gastric	15	30	3	1	6	3
duodenal	18	30	2	2	4	2

No parasites were detected. Most isolates were oral Gram positive cocci and *Candida albicans*. Conclusion: This pilot study shows that asymptomatic HIV+ pts presented BO more frequently (29%) than HIV- controls (9%). No fecal bacteria were found in BO pts, suggesting a descending route of infection. Further studies are needed to assess the role of BO in asymptomatic HIV pts as well as in those with diarrhea and/or weight loss.

EFFECT OF OLSALAZINE ON BILE ACID UPTAKE IN RAT ILEUM AND BRUSH BORDER MEMBRANE VESICLES. A Chawla, SG Koniaris, BL Shneider, GA Michaud, PI Karl, SE Fisher. Departments of Pediatrics, North Shore University Hospital-Cornell University Medical College, Manhasset, NY and Yale University, New Haven, CT

Olsalazine (OLZ), a relatively new form of 5-aminosalicylic acid (5-ASA), is being used for the treatment of colitis in inflammatory bowel disease. A major side effect of Olsalazine is profuse diarrhea (12% of patients), due, at least in part, to drug-induced enhancement of ileal water and electrolyte secretion. Another mechanism by which OLZ contributes to diarrhea may be an inhibition of bile acid (BA) uptake. OLZ is a prostaglandin inhibitor, and other prostaglandin inhibitors have been shown to block ileal BA uptake. OLZ-induced inhibition of ileal bile acid transport might result in excess bile acids reaching the colon, with consequent choleretic diarrhea. Therefore, we studied the effect of OLZ on rat ileal absorption of taurocholate.
METHODS: BA uptake was determined in rat ileal segments, everted sacs and brush border membrane vesicles (BBMV). BA uptake by ileal segments was determined in oxygenated Kreb's Ringer buffer (KRB, pH 7.4) containing 100μM ^{3}H-Taurocholate (Tc) at 37°C. Everted sacs were used to estimate transmucosal transport. Segments and everted sacs were treated with 5mM OLZ for 30 minutes prior to and throughout 10 min Tc uptake. BBMV were prepared from the rat terminal ileum using divalent cation precipitation. BBMV were used to define the direct effect of OLZ on Tc uptake kinetics.
RESULTS: At 5mM concentration, OLZ inhibited 10 minute Tc uptake by 69.4 ± 6.6 % (mean ± SEM) ($p<0.01$) (n=10 animals). Increasing concentrations of OLZ resulted in a dose dependent inhibition of Tc uptake. Ten-minute Tc uptake with 0.5, 1.0, 2.0, 2.5 and 5 mM OLZ was inhibited by 13.5, 39.6, 49.7, 66 and 70.5%, respectively. Transmucosal transport in everted sacs was also inhibited by OLZ (51.7 ± 13.3% inhibition relative to control; $p<0.01$). In BBMV, OLZ inhibited 45-second Tc uptake in a dose dependent manner. Tc increased the Km, but not the Vmax, suggestive of competitive inhibition of Tc uptake by OLZ.
CONCLUSION: OLZ inhibits Tc uptake and transmucosal transport in the rat ileum. Inhibition by OLZ is dose-dependent. This effect of OLZ on ileal function may contribute to the diarrhea observed with the drug.

PROTECTIVE IMMUNIZATION AGAINST HELICOBACTER. THE NEED FOR STIMULATION OF THE COMMON MUCOSAL IMMUNE SYSTEM. M Chen, A Lee, S Hazell, P Hu, Y Li. School of Microbiology and Immunology, University of New South Wales, Sun Yat Sen University, First People's Municipal Hospital, Guangzhou, People's Republic of China.

Previous work using an animal model had shown it was possible to protect against challenge with living helicobacters by oral immunisation using a cholera toxin adjuvant. The aim of this experiment was to investigate the humoral and mucosal immune response of animals immunised by various routes and correlate these responses with the level of protection against oral challenge. Measurement of biliary IgA was taken as a measure of stimulation of the mucosal immune system.
Methods Specific pathogen free, Balb/C mice were immunised by the following routes: *Oral*. Saline, sonicate of *H. felis*, sonicate of *H. felis* plus 10μg cholera toxin or 10μg cholera toxin alone. (Days 1,3,6,30,54). *Intravenous* 10^8 viable cells *H. felis* (Weeks 1,2,4,8,12). *Intraperitoneal* 10^8 viable cells *H. felis* (Weeks 1,2,4,8,12). All animals were challenged with 10^8 viable *H. felis* via oro-gastric tube post immunisation (Oral 10 days pi, i/v and i/p 2 months pi). Three weeks after challenge, infection with *H. felis* was assayed. Humoral immunity was assayed using an IgG ELISA on serum samples. Mucosal immunity was assayed using an IgA ELISA on pooled bile samples.
Results

Route of immunisation	Vaccine	No of animals	Protection %	Humoral immunity #	Mucosal immunity #
Oral	saline	21	0	3.0±0.3	3.3±0.2
Oral	*H. felis*	21	0	3.2±0.2	3.3±0.3
Oral	*H. felis* + cholera toxin	23	96	3.4±0.2*	4.1±0.2*
Oral	Cholera toxin	23	9	3.1±0.3	3.4±0.2
Intraperitoneal	*H. felis*	22	55	6.2±0.3*	3.8±0.1*
Intravenous	*H. felis*	20	0	4.5±0.2*	3.6±0.2*

ELISA unit (log 10): * $p<0.05$.
Conclusion Oral immunisation with a sonicate of *H. felis* and cholera toxin adjuvant induced nearly complete protection against oral challenge. Intraperitoneal hyperimmunisation with living *H. felis* also induced significant but not complete protection. Hyperimmunisation via the intravenous route induced no protective immunity. Protection correlated with the level of anti *H. felis* IgA antibody detected in the bile but not with humoral IgG. Stimulation of the common mucosal immune systen is essential for the induction of a protective immunity against gastric helicobacter infection.

BIOCHEMICAL CHANGES IN INTRACELLULAR MUCUS IN PATIENTS WITH GASTRITIS BEFORE AND AFTER ERADICATION OF *HELICOBACTER PYLORI*. C.P. Cheney, T. Shea-Donohue, C.L. Maydonovitch, R.A. Truesdale, P. Branton and R.K.H. Wong; Walter Reed AMC, Washington D.C. and USUHS, Bethesda MD.

The human gastric mucus layer has long been considered a protective barrier. *Helicobacter pylori* (HP) infection of human gastric antrum has been associated with thinning of this protective mucus layer and it has been speculated that HP does this by secretion of enzymes that can modify this mucus layer. Purpose: To determine if eradication of HP in patients with gastritis affected the carbohydrate (CHO) composition of intracellular gastric mucus. Methods: Antral biopsies were obtained in 13 patients with endoscopic gastritis who had HP infection documented by histologic examination and/or CLO test. HP was eradicated by using a 2 week protocol of peptobismol, tetracycline, and metronidazole. Repeat antral biopsies were obtain six months later (mean 206 days). Changes in CHO composition of intracellular mucus in gastric surface epithelial cells (SEC) versus gastric gland cells (GGC) in HP infected versus HP eradicated patients was assessed using horseradish peroxidase labeled-lectin binding to formalin fixed tissue. The presence of terminal fucose (Fuc) was assessed by the lectin UEA whereas wheat germ agglutinin assessed the presence of N-acetyl-glucosamine (NGlu). The lectin binding was assessed under light microscopy @ 400X and scored from 0 to +4 by 2 blinded observers.
Results:

	Fuc		NGlu	
	SEC	GGC	SEC	GGC
HP infected	2.06±.91	1.77±.88	2.81±.57	1.88±.88
HP eradicated	2.50±.89	2.25±.95	3.34±.41	2.08±.83
P values	0.27	0.22	0.019*	0.58

Conclusions: 1.) These results suggest that following HP eradication, there is indirect evidence for a trend toward increase in mucus in the SECs and GGCs. 2.) This increase in mucus is significantly greater for NGlu in the SECs. 3.) HP infection may inhibit the overall production of mucus as previous studies in our lab have shown similar decreases in soluble mucus in HP infected patients (Gastro 100:A167,1991).

STIC-ILL

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Title: PROTECTIVE IMMUNIZATION AGAINST HELICOBACTER - THE NEED FOR STIMULATION OF THE COMMON-MUCOSAL IMMUNE-SYSTEM
Author(s): CHEN M; LEE A; HAZELL S; HU P; LI Y
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Helicobacter-Specific Cell-Mediated Immune Responses Display a Predominant Th1 Phenotype and Promote a Delayed-Type Hypersensitivity Response in the Stomachs of Mice[1]

Marjan Mohammadi,* Steven Czinn,† Ray Redline,* and John Nedrud[2]*

Studies regarding the nature of cell-mediated immunity in *Helicobacter pylori* infection and its role in pathogenesis have yielded controversial results. To address this issue in a controlled manner, we have employed the well-characterized *Helicobacter felis*-mouse model. Immunized/challenged and nonimmunized/infected mice were evaluated for cellular proliferation, gastric inflammation, and cytokine and Ab production at various times after infection. We observed two types of cell-mediated immune responses depending on the nature of the Ag preparation. The first response is a *Helicobacter*-independent response, present in all experimental groups, which is directed toward Ags such as urease and heat shock proteins. The second is a *Helicobacter*-dependent cellular response restricted to mice previously exposed to *Helicobacter* Ags either by immunization or infection. This response was not seen in noninfected controls. The *Helicobacter*-dependent cellular response had a Th1 phenotype, as either infected or immunized/challenged mice demonstrated local and systemic production of IFN-γ and undetectable levels of IL-4 or IL-5. Cellular proliferation correlated with the severity of gastric inflammation in both immunized/challenged (protected) and nonimmunized/infected mice. Finally, in vivo neutralization of IFN-γ resulted in a significant reduction of gastric inflammation in *H. felis*-infected, as well as immunized/challenged, mice. This treatment also revealed the presence of Th2 cells, restricted to immunized/challenged mice, as demonstrated by local and systemic production of IL-4 in these mice. These data demonstrate that *Helicobacter* infection and/or immunization stimulate a predominantly Th1-type, Ag-specific response and promote a local delayed-type hypersensitivity response in the stomach that may be inhibited by depletion of IFN-γ. *The Journal of Immunology*, 1996, 156: 4729–4738.

Helicobacter pylori infects the gastric mucosa of half of the adult population worldwide. *H. pylori* infection is the primary cause of antral gastritis and is associated with the majority of duodenal ulcers (1–4). There is an increased risk of gastric cancer and mucosa-associated lymphoid tissue lymphomas associated with *H. pylori* infection (5–7). Despite the presence of high titers of *Helicobacter*-specific Abs in the serum and gastric mucosa of *H. pylori*-infected patients (8, 9), they remain chronically infected and are unable to clear the infection. To date all of the studies on cellular immune responses to *H. pylori* have been performed in humans and the nature of Ag-specific T cell-mediated responses has not been well defined. In particular whether pro-inflammatory Th1 or anti-inflammatory Th2 responses predominate in *Helicobacter* infection and how these responses may contribute to immunity versus disease is unclear. Some investigators have provided evidence for priming of an *H. pylori*-specific cellular response in *H. pylori*-positive individuals versus *H. pylori* negative controls (10, 11). In support of this, a recent study has reported spontaneous IFN-γ production by mucosal lymphocytes from *H. pylori* positive patients with gastritis (12). In contrast, some investigators have reported activation of cellular responses in both *H. pylori*-infected and noninfected individuals, with a variably reduced response in infected patients (13–17). These conflicting results may be partly attributed to the large number of variables involved in the human studies, including different strains of infecting organisms, variable durations of infection, different Ag preparations used for in vitro stimulation, and different host factors such as genetic backgrounds, medications, diet, and stress.

Here we demonstrate two types of anti-*Helicobacter* cellular responses. One is a *Helicobacter*-independent cellular response, defined as a response seen in all experimental groups including the noninfected, naive mice. This response is directed toward urease, heat shock proteins, and possibly other Ags in the heat-inactivated bacterial preparation. The second is a *Helicobacter*-dependent cellular response, defined as a response seen only in *Helicobacter felis*-immunized or infected mice, induced by Ags whose nature is yet undefined. This latter response has a predominantly T helper 1 phenotype. This Th1 response promotes a delayed-type hypersensitivity response in the stomachs of mice, which is abrogated by in vivo neutralization of IFN-γ. Furthermore, suppression of the Th1 response in the immunized mice unmasks the presence of a subpopulation of Th2 cells. This treatment, however, does not affect the magnitude of infection (in infected mice) or the rate of protection (in the immunized mice).

Materials and Methods

Animals

Murine pathogen-free, female C57BL/6 mice (6- to 8-wk-old) were obtained from Taconic Laboratories (Germantown, NY) and were housed in microisolator cages with free access to autoclaved chow and water. The Case Western Reserve University animal facility is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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ddress correspondence and reprint requests to Dr. John Nedrud, Department Pathology, Case Western Reserve University, Biomedical Research Building, 901, 10900 Euclid Avenue, Cleveland, Ohio 44106.

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Bacteria

H. felis were recovered from gastric biopsy specimens of a cat and identified as *H. felis* based on morphology, Gram stain, and the production of urease, catalase, and oxidase. Organisms were stored in 50% PBS, 25% glycerol, and 25% heated FCS at −70°C (18).

Bacterial antigens

H. felis organisms were inoculated onto Columbia agar containing 7% horse blood and incubated microaerobically at 37°C for 5 to 7 days.

Whole cell sonicate. The organisms were harvested in PBS and the resulting suspension was sonicated to lyse bacteria at 4°C, cleared of cellular debris by low speed centrifugation, and sterile filtered. The protein concentration of whole cell sonicate was determined by the method of Lowry (19), and aliquoted and stored at −70°C until needed for oral immunizations or in vitro stimulation of lymphocytes. Heat-inactivated *H. felis* was prepared by heating the harvested *H. felis* organisms for 1 h at 100°C. Optimal density of this preparation was measured at OD_{560} and used at a final OD of 0.1 (17).

Outer membrane proteins. Bacterial suspensions were treated with 1 mg of RNase and DNase in 0.5 M Tris-EDTA buffer at 4°C immediately before sonication and low speed centrifugation as described above. Bacterial envelopes were separated from the cleared lysate by ultra centrifugation at 150,000 × *g* for 1 h. Outer membranes were then separated from the cell envelopes by differential solubilization in sodium *n*-lauroyl sarcosine and recovered by ultra centrifugation. The resulting pellet was then resuspended in 0.05 M phosphate buffer and protein concentration was determined by the method of Lowry (19). Recombinant *H. pylori* urease was a generous gift from Oravax Inc., Cambridge, MA. Mycobacterial heat shock proteins 65 and 71 K_d were kind gifts from Dr. W. H. Boom (Case Western Reserve University, Cleveland, OH). The optimal concentration for *H. felis* sonicate, *H. pylori* urease, mycobacterial heat shock proteins, and anti-CD3 Ab (PharMingen, San Diego, CA) was determined to be 1 μg/ml. Hen egg lysozyme (Sigma Chemical Co., St. Louis, MO) was used as an irrelevant Ag for in vitro studies at the same concentration.

Immunization and infection

Mice were lightly sedated by CO_2 inhalation before intragastric immunization. The whole cell sonicate plus cholera toxin were suspended in 0.2 M $NaHCO_3$. One-half milliliter of this preparation containing 4 mg Ag and 10 μg cholera toxin was delivered to the stomachs of mice by intubation through polyethylene tubing attached to a hypodermic syringe. This procedure was repeated four times over a period of 1 mo. The immunized mice along with the nonimmunized mice were challenged 7 days after the last immunization by oral administration of 5×10^7 cfu *H. felis*. *H. felis* were quantitated as previously described (20). Mice were considered infected by either a positive urease test or direct staining of bacteria in Giemsa-stained histologic sections. The four following groups of mice were used in the present study: 1) naive (nonimmunized/nonchallenged) mice, 2) infected (nonimmunized/infected) mice, 3) immunized/nonchallenged mice, and 4) protected (immunized/challenged).

Serum antibody titers

Blood was obtained from mice before killing and serum was collected. *H. felis*-specific Ab titers were determined by ELISA as previously described (18). Briefly, microtiter plates were coated with *H. felis* outer membrane proteins, incubated overnight at 4°C, washed, and blocked with 1% BSA/PBS at room temperature for 1 h. Serial dilutions of the sera were then applied and incubated for 90 min at room temperature. The colorimetric assay used alkaline phosphatase-conjugated goat anti-IgG, anti-IgG1, or anti-IgG2a antisera as conjugate and *P*-nitrophenol phosphate as substrate. Plates were read at an optical density of 405 nm in an automated ELISA plate reader (BIO-TEK, EL309). The serum Ab levels were either plotted as the optical density at 405 nm or as end point titers, defined as the highest dilution (depicted as reverse $\log_{10}$ dilutions) giving an OD_{405} reading of 0.05 above the conjugate control (21).

Lymphocyte proliferation

Spleens were removed from mice at death and gently disrupted. Single cell suspensions were adjusted to 10^6 cells/well in 96-well microtiter plates in HL-1 medium (Hycor, Portland, ME), containing 2 mM L-glutamine with or without the optimal concentration of Ag or mitogen in triplicates or quadruplicates. The cells were incubated for 3 and 5 days and pulsed with 1 μCi [^{3}H]thymidine (ICN, Irvine, CA) per well for the last 16 h of incubation. Thymidine incorporation was measured by liquid scintillation. The stimulation index was calculated by dividing the counts obtained from Ag-stimulated cells by the counts obtained from unstimulated (media incubated) cells. Gastric lamina propria lymphocytes were isolated as p viously described for mouse intestines (22). The stomachs of mice were into 5-mm pieces, washed, and subjected to EDTA digestion. This allow separation of intraepithelial lymphocytes from the tissue and the remaini tissue was subjected to collagenase digestion leased lamina propria lymphocytes from gastric tissue, which were subs quently cultured in the same manner as the spleen cells.

Cytokine production and measurement

Cells prepared as described above were incubated in 24-well plates at 5 10^6 cells/ml with or without optimal concentrations of Ag and mitogen f 48 and 72 h. The supernatants were then harvested and kept at −70° before cytokine measurements. The presence of IL-4, IL-5, and IFN-γ w determined by sandwich ELISA using nonbiotinylated and biotinylate mAbs for each cytokine as primary and secondary Abs, respectively. Thes Abs were as follows: IFN-γ, R46A2 and XMG1.2; IL-5, TRFK5 an TRFK4; and IL-4, BVD-4 and BVD-6 (PharMingen). Wells of 96-we microtiter plates were coated overnight at 4°C with the primary Ab (μg/ml) and blocked with 1% gelatin in PBS-Tween. Culture supernatant and serial dilutions of recombinant mouse IFN-γ, IL-4, and IL-5 (Phar Mingen, as standards) were applied and incubated overnight at 4°C. Bi otinylated secondary Abs were added (1 μg/ml) for an additional 4 h a room temperature. Streptavidine alkaline phosphatase was used as the conjugate and *P*-nitrophenol phosphate as the substrate. The optical density was measured at OD_{405} in an automated ELISA plate reader (BIO-TEK, EL309). The sensitivity of these assays was determined to be 80 pg/ml.

Histologic evaluation

Strips of the entire greater curvature of the stomach of mice were cut, fixed in 10% buffered formalin, and embedded in paraffin. Five-micron sections were cut and stained with hematoxylin and eosin and Giemsa reagent. Gastric sections were evaluated in a blinded fashion according to the following criteria.

Intensity of inflammation. Overall intensity of inflammation in the worst ×10 microscopic field was scored on a scale from 0 to 5 based on the following criteria: Grade 1 = rare inflammatory cells; Grade 2 = multiple clusters of inflammatory cells; Grade 3 = diffuse inflammation of variable intensity with architectural disruption; Grade 4 = diffuse inflammation, uniformly severe, without architectural disruption; Grade 5 = diffuse inflammation, uniformly severe, with architectural disruption.

Extent of inflammation. The percentage of the mucosal surface involved by inflammation was ranked as follows: Grade 0 = None; Grade 1 = <25%; Grade 2 = 25 to 50%; Grade 3 = 50 to 75%; Grade 4 = >75%. In some experiments, overall grade of inflammation was qualitatively graded based on a combination of intensity and extent of inflammation.

Character of inflammation. The following cellular components of the inflammatory infiltrate were graded qualitatively from 0 to 3 corresponding to mild, moderate, and severe, respectively, as also described previously (36): mononuclear cells, polymorphonuclear cells, and plasma cells.

***Quantitation of* H. felis.** Extent of infection was estimated by the average number of H. felis positive glands/centimeter observed in Giemsa-stained histologic sections. Both fundus and antrum of the stomach were scored in this manner and averaged to give one value for bacterial infection of each mouse.

Anti-IFN-γ treatment

Neutralizing anti-IFN-γ mAb (XMG1.2) and the hybridomas were generously provided by Dr. Fred Heinzel (VA Medical Center, Cleveland, OH). Immunized and/or nonimmunized mice were i.p. injected with 1 mg of XMG1.2 or control rat IgG (Sigma Chemical Co.) 1 day before oral inoculation with *H. felis* and 10 days after challenge. All groups were killed 5 wk after challenge and evaluated for spleen and gastric cytokine production, serum Abs, gastric pathology, and magnitude of infection.

Statistical analysis

Comparison of cellular proliferation, IFN-γ production, grade of inflammation, and rate of bacterial colonization among experimental groups was made by analysis of variance with Fishers' protected *t* test and the Mann Whitney nonparametric test. Correlation analysis was performed by simple regression using the StatView II program.

STIC-ILL

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But other medical religions there are in abundance. They try to be modern and to think in paramedical terms, just as 'creation-science' invokes geophysical and astronomical concepts. Thus homeopathy and naturopathy utilise vitamins and minerals in supposedly boosting natural resistance to disease. Acupuncture tries to protect itself within a coating of gate theory of pain plus endorphins. Such would be more impressive if there was evidence that pin pricks in ear lobes are more effective than the witch doctor's scratch marks over the liver. Anthroposophy (Rudolf Steiner) tries to ground itself in physiology. Reflexology does not even try. Some medical religions are very close to OM and physiotherapy:osteopathy and chiropractic.

An intriguing phenomenon is the espousing of one or other medical religion by scientifically trained physicians, but perhaps this is not so odd; after all, a medical training does not immunise one against the irrational or religious impulse.

These medical religions are indeed complementary and holistic in the ancient sense, and they are inescapably part of the company of the sick, the suffering and the dying. Their historical momentum is far too powerful to be dismissed as irrational nonsense. In one form or another the religions of medicine will continue to accompany us and people will find comfort in them and supply anecdotal evidence of their value. But the religions of medicine must remain on the outside; they have no place in a medicine committed to the scientific method.

A personal note is in order: my mother died at the age of 67 years from Parkinson's disease, immobility and cardiac failure. At the time my father suggested the attentions of a healer he had heard about, and whose hands, or herbs (or was it some gadget?) supposedly worked wonders. I forbade it, preferring to spare her further complementary and holistic distress and disappointments. She died at home, in my presence, and with peace of mind.

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Impact of a rapid antenatal Rh test on laboratory testing

To the Editor: Prevention of haemolytic disease of the newborn by administering anti-D immunoglobulin to rhesus (Rh)-negative women is part of standard antenatal care.

Until a convenient and rapid test for Rh was introduced in 1993, samples of blood from approximately 175 000 pregnant women were submitted each year to the Natal Blood Transfusion Service (NBTS) for Rh typing and red cell antibody screening. Many patients were from rural areas, making transport of samples, collation of reports and follow-up difficult. Rh antibodies (anti-D) were detected in 173 patients during 1992; delivery samples were only received from 59 of them.

The RAPIDTEST Rh kit (available from the NBTS, PO Box 2356, Durban, 4000) was introduced to offer a cost-effective Rh antenatal service at the primary health care level. The test is carried out at the clinic on a finger-prick sample, and the Rh type of the patient can be determined immediately.[1] It is recommended that venous samples be taken from all patients with a poor history and those who test Rh-negative by the RAPIDTEST Rh method. Samples should be sent to an appropriate laboratory for red cell antibody tests.

The RAPIDTEST Rh was introduced throughout the Natal/KwaZulu area during January - March 1993.[2] By June 1993, the number of samples submitted to the NBTS for Rh typing and antibody tests had fallen from a monthly average of 14 583 in 1992 to 1 392 (Fig. 1). During the 3 months April - June 1993, 27 cases of anti-D were identified, which compares favourably with 32 cases during the same period in 1992. The difference was that the 27 in 1993 came from only 5 058 screened samples whereas 46 112 samples had to be tested in 1992. The RAPIDTEST Rh kit procedure therefore seems effective in identifying patients at risk for Rh.

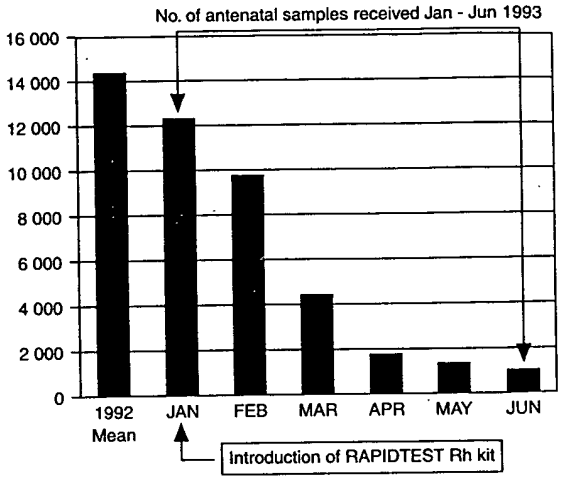

FIG. 1.
NBTS monthly antenatal statistics, 1993.

The programme for the prevention of haemolytic disease of the newborn is funded by the Subdirectorate Genetic Services, Department of National Health and Population Development. The introduction of the RAPIDTEST Rh in Natal during the first quarter of 1993 has reduced the cost by one-third. This amounts to a total saving of R95 548 in just 3 months.

The advantage of immediate identification of patients at risk for Rh, together with the low cost of the RAPIDTEST Rh, permits a very cost-effective Rh antenatal service.

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Autogenous vaccination against *Helicobacter pylori*

To the Editor: Evidence for a major pathogenic role for *Helicobacter pylori*, formerly known as *Campylobacter pylori*, is mounting slowly. It has been established that the organism is the main cause of chronic active gastritis.[1] It is also associated with peptic ulceration, particularly duodenal ulceration, where it undoubtedly plays a part in the chronicity of the condition. It is generally accepted that eradication of the organism reduces the relapse rate in duodenal ulceration.[2] Through causing gastritis, which in turn may cause atrophy of the gastric mucosa, the organism may be implicated indirectly in the causation of carcinoma.[3,4] It also allegedly causes halitosis.

H. pylori itself is Gram-negative, micro-aerophilic and can survive in low-pH secretions. It is infectious; volunteers exposed to it develop gastritis. Once established within the gastric mucosa it is known to persist for many years, even a lifetime.

Treatment, with the aim of eradication of the organism, consists of bismuth in varying combinations with amoxycillin, metronidazole or tetracycline for 2 - 4 weeks. However, the organism appears to be becoming more resistant to these drugs; at the Boksburg-Benoni Hospital

branch laboratory of the South African Institute for Medical Research, for example, 1 in 3 cultures grown recently appeared to be completely resistant to metronidazole.

This increasing resistance and the difficulty in eradicating the organism completely led to the concept of vaccination against *H. pylori*.

Vaccination treatment was considered for a patient with active chronic gastritis of very long standing which did not respond adequately to treatment including a 3-week course of bismuth and amoxycillin; the patient, who happened to be a medical practitioner, gave his full and enthusiastic co-operation.

The patient, in his 70s, had suffered pain and discomfort intermittently for approximately 45 years, during which time various investigations revealed no significant abnormality and no specific treatment was given.

In 1987 the patient consulted me, and gastroscopy on 30 December revealed gastritis with increased inflammatory change in the antrum and on the gastric surface of the pyloric ring. Histological examination of biopsy specimens taken from the antrum revealed chronic active gastritis with micro-organisms resembling *H. pylori*. The urease test was immediately positive for *H. pylori*. The patient was treated with bismuth together with amoxycillin for 3 weeks with some improvement of his symptoms, but his discomfort soon returned.

Findings on repeat gastroscopy on 18 November 1991 were similar to those of the first examination except that the rim of the pyloric ring appeared to be even more inflamed. The urease test was again positive and *H. pylori* was easily identified histologically. Biopsy specimens were taken for culture and *H. pylori* was grown on chocolate agar at 37°C under micro-aerophilic conditions for 5 days.

Ten plates were harvested for vaccine production. The suspension was treated at 60°C for 6 hours to inactivate the organism. It was checked for sterility. Phenol was added to 0,5%. It was then standardised to obtain 0,5 × 10^6 organisms per millilitre in the 0,5% phenol in saline. It was finally checked for purity and sterility.

The course of vaccination commenced on 25 May 1992, starting with 0,05 ml of this autogenous vaccine given subcutaneously. The injection was administered every 5 days in increasing dosage. There was a slight skin reaction initially, but generally the patient experienced no discomfort from the vaccination. The course of injections lasted approximately 2 months, at the end of which the patient reported improvement of his gastric symptoms. A third gastroscopy was performed soon after the completion of the course of vaccination and this time no evidence of any significant gastritis could be detected. Both the urease test and culture were now negative. 'A mild gastritis with mild activity' was reported, however, and a few organisms resembling *H. pylori* were seen histologically. The patient's immunoglobulin values remained normal throughout, no response being recorded after vaccination. In experiments in which mice were given an oral vaccine, a positive immunoglobulin response was recorded.[5]

Serological tests such as the enzyme-linked immunosorbent assay (ELISA)[6] would have confirmed active *H. pylori* infection and possibly shown an increased immunisation status after vaccination. However, this test was not available at our laboratory. Since the degree of immunity induced by the vaccine was unknown, the patient was given 4 booster doses of vaccine at 3-monthly intervals, receiving the last one on 31 July 1993. Follow-up gastroscopy on 26 August revealed very mild gastritis and no congestion of the pyloric region. The urease test was again negative (cultures were not done).

In conclusion, vaccination appeared to improve the patient's clinical condition as well as the gastroscopic appearance, while the urease test and culture became negative. The significance of the few organisms reported is difficult to assess, but I would postulate that their pathogenicity has been inhibited. Further follow-up of this case is of utmost importance to evaluate the efficacy of the vaccine more fully, as is a further study involving a large number of cases in a therapeutic trial by vaccination.

With *H. pylori* showing increasing resistance to antibacterials, the frequency of relapses even after antibacterial treatment and the general difficulty in eradicating the organism once it is established, vaccination may become the treatment of choice for this infection and its effects on the gastro-intestinal system.

I thank Professor K. Klugman for his kind assistance with the culture media, Dr J. Southern for producing and supplying the autogenous vaccine, and for his friendly co-operation, and Denise Clough and Pia Duffy of the Boksburg-Benoni Hospital Branch Laboratory of the South African Institute for Medical Research for their enthusiastic and skilful technical assistance with the bacterial cultures and various tests.

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Minimally invasive thoracic surgery

To the Editor: Over the last 2 - 3 years endoscopic abdominal surgery and in particular endoscopic cholecystectomies have increased significantly, with endoscopic cholecystectomy being the operation of choice for acute cholecystitis. Minimally invasive thoracic surgery has now become a reality with numerous procedures being successfully performed in the chest.[1-3]

Over the last year 76 endoscopic thoracic procedures have been performed in my unit. These include 26 bullous ligations and pleurodesis, 14 pleural toilets, 15 lung biopsies, 11 pleural biopsies, 2 hilar gland biopsies, 7 bilateral trans-axillary sympathectomies, and 1 division of an anomalous subclavian artery.

All these patients would otherwise have required a full thoracotomy. Subsequent thoracotomy was avoided in 57 of the first 60 cases. During the learning phase 3 patients required thoracotomy because of difficulties in performing the procedure.

Thorascopic treatment used as a first-line measure in various thoracic diseases is a safe and relatively atraumatic procedure which does not exclude subsequent more invasive surgical procedures and in many cases avoids the morbidity attached to a full thoracotomy. The benefits to the patient are an early return to work and a significant decrease in long-term pain. Experimental work on endoscopic oesophagectomies and lobectomies has been reported. This is an exciting field of research.

Minimally invasive surgery is an important adjunct to the armamentarium of the modern surgeon and as such has a distinct place in thoracic surgery.

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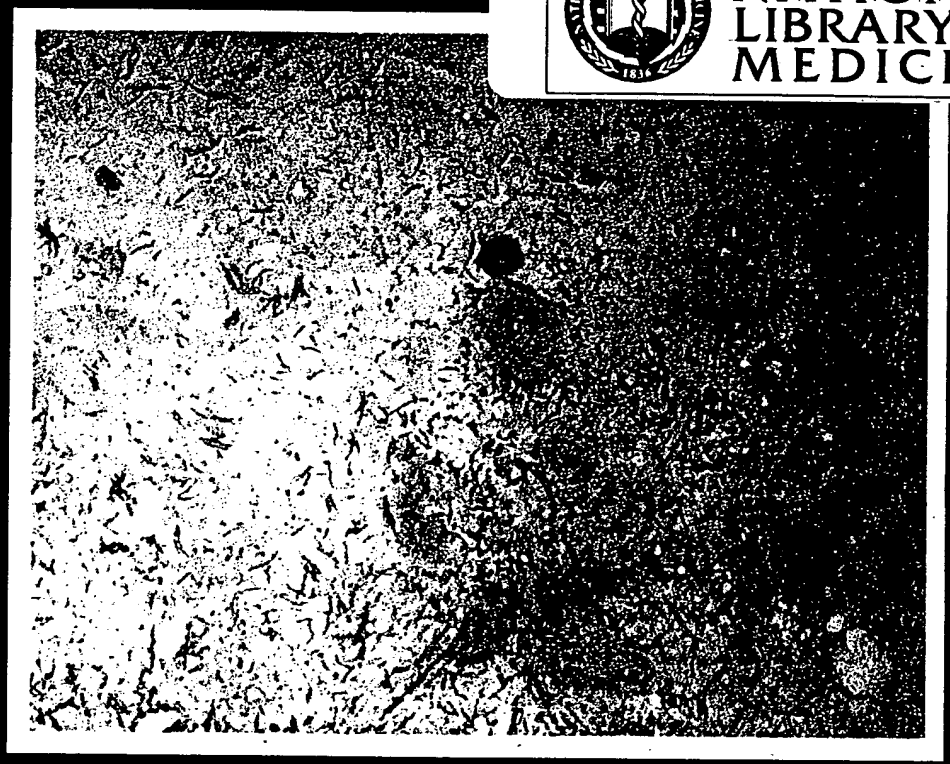

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